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Generally applicable methods to purify intracellular coccidia from cell cultures and to quantify purification efficacy using quantitative PCR

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Abstract

The objective of this study was to evaluate the utility of a simple, efficient, and rapid method for the isolation of *Sarcocystis neurona* merozoites and *Besnoitia darlingi* tachyzoites from cultured cells. The efficacy of this purification method was assessed by microscopy, SDS-PAGE, Western blotting, immuno-fluorescence, and three novel quantitative PCR assays. Culture medium containing host cell debris and parasites was eluted through PD-10 desalting columns. This purification method was compared to alternatives employing filtration through a cellulose filter pad or filter paper. The estimated recovery of *S. neurona* merozoites purified by the column method was 82% (±3.7) of the original merozoites with 97.5% purity. In contrast, estimated recovery of *S. neurona* merozoites purified by filter pad and filter paper was 40% and 30% with 76% and 83% purity, respectively. The same procedures were applied to purify *B. darlingi* tachyzoites from cultured cells. Of the original cultured *B. darlingi* tachyzoites, 94% (±2.5) were recovered from the PD-10 column with 96.5%, purity whereas percentage recovery of *B. darlingi* tachyzoites purified by filter pad and filter paper were 51% and 35% with 84% and 88% purity, respectively. All described methods maintained sterility so that purified parasites could be subsequently cultured in vitro. However, purification using a PD-10 column minimized parasite loss and the loss of viability as determined by the trypan blue dye exclusion assay, the rate of parasite production, and plaque forming efficiency in cell culture. Moreover, column-purified parasites improved the sensitivity of an immuno-fluorescent (IFA) analysis and real-time quantitative PCR assays

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targeted to parasite 18S ribosomal DNA and hsp70 genes. This technique appears generally applicable for purifying coccidia grown in cell cultures.

Keywords: Apicomplexa; PD-10 column; Purification; Real-time quantitative detection; *Sarcocystis neurona*; Taqman PCR; Tissue culture

1. Introduction

Sarcocystis neurona is the primary causative agent of equine protozoal myeloencephalitis (EPM) in the Americas (Mackay et al., 1992; Dubey et al., 2001b). *S. neurona* was first described in 1991 after its isolation from a horse (Dubey et al., 1991). It belongs to the phylum Apicomplexa, a large group of mainly intracellular parasites. *Besnoitia darlingi* is an Apicomplexan parasite employing opossum (*Didelphis virginiana*) as an intermediate host and cat as a definitive host (Dubey et al., 2002). Equine dermal cells support the development of *S. neurona* in cell culture (Mansfield et al., 2001). In addition, bovine monocytes, equine kidney cells, cardiopulmonary endothelial cells, deer testes, vero cells, African green monkey (*Cercopithecus aethiops*) kidney cells (CV-1), rat myoblasts, and bovine turbinate (BT) cells have been employed successfully to support *S. neurona* replication (Dubey et al., 2001b). Bovine monocytes, CV-1 cells, and BT cells also have been used to cultivate and maintain *B. darlingi* tachyzoites (Dubey et al., 2002; Elsheikha et al., 2005).

One of the major obstacles to research with *S. neurona* and other coccidia is the difficulty of obtaining abundant parasites free of host cell contaminants. Such pure parasite suspensions would facilitate studies of host–parasite interaction and are needed in order to extract protein and genetic materials for biochemical, immunological, and molecular analyses. Difficulty in obtaining sufficient numbers of *S. neurona* merozoites free of contaminating host cells or cell debris is generally recognized. Approaches have been reported for separating the parasite from host cells. Dubey et al. (2001a) reported the isolation of merozoites from cell culture by filtration through a syringe filter. However, the syringe filters tend to clog easily. Lindsay and Dubey (2001) passed the merozoite suspension through a 27-gauge needle attached to a 10 ml syringe in order to rupture host cells, and then passed the suspension through a sterile 3 μm filter to remove cellular debris. However, the parasite yield is low using this method. In another study, a calcium ionophore (A23187) was used for synchronous release of parasites from host cells (Ellison et al., 2001). This method was slow, complicated for large-scale preparation and resulted in preparations that contained appreciable host cell contamination. Although useful, none of these approaches are well suited for producing an abundance of pure merozoites desirable for immunological, biochemical, and genetic analyses.

Quantitative real-time PCR provides a sensitive, accurate and fast method for estimating the abundance of DNA templates by monitoring the accumulation of fluorescent PCR products. In assays that approach 100% efficiency, in which a doubling of product occurs with each successive amplification cycle during the exponential phase, the number of cycles required to significantly exceed background fluorescence (C_t) is inversely proportional to the initial template concentration (Klein, 2002; Ginzinger, 2002).

Herein we describe the adaptation of a previously described approach (Hemphill et al., 1996) to isolate viable over 80% of the *S. neurona* merozoites and over 90% of the *B. darlingi* tachyzoites cultivated in bovine turbinate cells at a purity of over 95%, and describe new real-time quantitative PCR assays to estimate the relative and absolute concentrations of parasite and host DNA in such samples. Enrichment was achieved by eluting each parasite suspension through a PD-10 desalting column. The described method compares favorably to known alternatives in parasite recovery, purity, and viability. Additionally, the procedure improves the sensitivity of immuno-fluorescent staining and of diagnostic PCR amplifications.

2. Materials and methods

2.1. Parasite strains and tissue cultures

S. neurona strains MIH1 and SN3 were maintained in bovine turbinate (BT) cell monolayers. BT cells were originally purchased from American Type Culture Collection (ATCC, CRL-1390, Manassas, VA, USA). BT cells were used between passages 15–17 and maintained in 8–10 ml of complete culture medium using 25 cm^2 plastic tissue culture flasks. Cells were fed Eagle's minimum essential medium (EMEM) with Earle's salt supplemented with L-glutamine, 10,000 U ml^{-1} penicillin G sodium, 10,000 μg ml^{-1} dihydrostreptomycin, 250 μg ml^{-1} of amphotericin B, non-essential amino acids, 100 mM sodium pyruvate, and 5–10% heat inactivated fetal bovine serum (FBS) (Gibco-Invitrogen, Grand Island, NY, USA). Cells were incubated at 37 °C in 5% CO_2/95% air. The *B. darlingi* MIBD1 strain was initially obtained from cysts isolated from a naturally infected opossum (*D. virginiana*) from Michigan in 2002. Methods used for isolation and maintenance of this strain were previously described (Elsheikha et al., 2005). Parasite stocks were maintained for long periods by varying the FBS concentration as described previously (Speer et al., 1986). Stock cell cultures were also maintained by lifting the monolayers with trypsin–EDTA (0.25% trypsin; 1 mM EDTA·4Na, Gibco) once a week and transferring the cells to new culture flasks. Approximately 5×10^4 of *S. neurona* merozoites and 3×10^4 *B. darlingi* tachyzoites were used to inoculate new flasks of BT cells. The development of mature schizonts occurred in 3–5 days in both parasites after inoculation of the flasks.

2.2. Purification of S. neurona merozoites

S. neurona merozoites were harvested from their feeder cell cultures when about 60–80% of the BT host cells were lysed. Free merozoites were removed from the tissue culture flasks by collecting the medium supernatant. A large portion of the remaining merozoites was released from their host cells by adding few milliliters of EMEM to the culture flasks and gently moving the side of the flasks 2–3 times and the merozoites were then collected. The preparation containing merozoites and host cell debris was mixed thoroughly, divided into three equal aliquots, and submitted to purification procedures by PD-10 column, cellulose filter pad, or filter paper. All the purification procedures were performed inside a biological safety cabinet under sterile conditions.

2.3. Purification using a PD-10 column

The merozoite suspension was washed twice in a buffer solution by centrifugation at 1500 × *g* for 5 min at 4 °C. Buffer solutions used were either sterile 1× PBS or 1× Hanks' balanced salt solution (HBSS). The final pellet was resuspended in 2.5 ml of buffer solution and applied to a PD-10 column filled with sephadex G-25M (Amersham Biosciences, Piscataway, NJ, USA), previously equilibrated with approximately 25 ml of the same buffer. The parasites were eluted with 3.5 ml of the same buffer used in equilibration of the column. The eluted, purified merozoites were used for determination of recovery, purity, and viability as well as for evaluating the utility of parasite materials purified by this method for molecular analyses.

2.4. Purification using cellulose filter pad and filter paper

The cellulose filter pad method for purifying the merozoite suspension required a glass filter assembly (Millipore, Bedford, MA, USA) (Fig. 1). A 47 mm glass holder apparatus was designed to handle large volumes of liquids. An autoclavable cellulose filter pad (cat. no. AP1004700, Millipore) was initially rinsed with sterile 1× PBS, pH 7.2 or EMEM immediately prior to use. Then, the merozoite suspension was passed through the filter pad and the filtrate was collected. The filtrate was centrifuged at 1500 × *g* for 5 min at 4 °C. The pellet was resuspended in 3.5 ml of 1× PBS. The filter paper method was performed identically using the same conditions, but by using Whatman filter paper (cat. no. 1001125, VWR International, West Chester, PA, USA) instead of a filter pad. The purified merozoites in both cases were used for determination of recovery, purity, and viability.

2.5. Determination of merozoites' yield and purity

In all experiments, the yield of merozoites was determined microscopically by counting individual

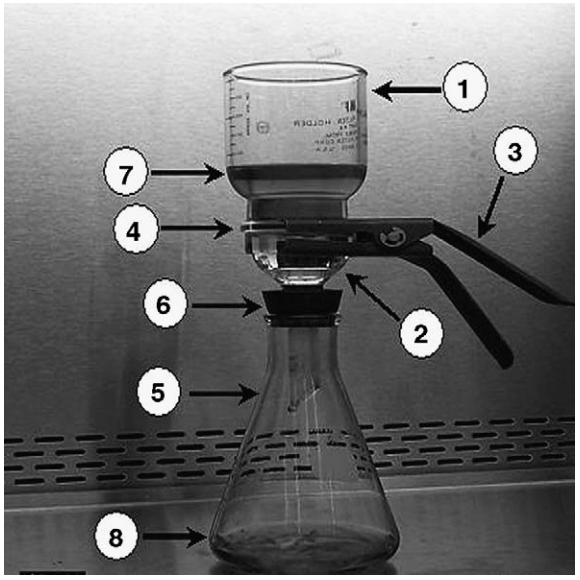

Fig. 1. Illustration of the filter assembly. It consisted of a removable 250 ml borosilicate glass funnel (1) and support base (2). An anodized aluminum spring clamp (3) sandwiched a cellulose filter pad or filter paper (4) between funnel and support base. Connection to 500 ml Erlenmeyer filter flask (5) is made with a silicone stopper (6). Parasite suspension with debris (7) and relatively pure parasite suspension (8).

intact merozoites before and after purification using the four outside squares and one central square of a standard hemocytometer chamber (Sigma, Saint Louis, MO, USA) at 200× magnification on a light microscope (Reichert-Jung, Pegasus Scientific, Frederick, MD, USA). The total number of merozoites/ml was estimated by averaging the counted squares and corrected for dilution and volume using the formula (total merozoites counted in 5 squares/5) $\times 10^4$ = merozoites/ml. Tubes containing the purified merozoites in 3.5 ml aliquots were vortexed for 10 s before counting. The purity of the final merozoite preparation was evaluated microscopically by differential counting of intact motile merozoites versus any other recognizable particles or cellular debris. Percentage purity was quantified by comparing the number of merozoites to the number of debris particles before and after purification.

2.6. Assessment of merozoite viability

The viability of the purified merozoites was assessed by two means: (1) trypan blue dye exclusion assay and (2) by evaluating the rate of *S. neurona* merozoite production and plaque forming efficiency in BT cell culture. Equal numbers ($\sim 12 \times 10^5$) of crude merozoites and merozoites purified by PD-10 column, filter pad, or filter paper were inoculated onto T-25 flasks containing a monolayer of BT cells in complete EMEM. The cultures were incubated at 37 °C in 5% CO_2. T-25 tissue culture flasks were mock-inoculated with complete EMEM and served as negative controls. Media was changed 36 h post-inoculation to remove extracellular merozoites and then at 3–4 days intervals thereafter. The inoculated cultures were monitored daily using a inverted light microscope (Carl Zeiss, Opton, Columbia, MD, USA) to follow the development of plaques and production of merozoites. The numbers of *S. neurona* plaques (infected foci) were estimated 10 days post-infection (DPI) using an inverted microscope, by counting the number of visible plaques per microscopic field at 200× magnification. These counts were expressed as a mean (±S.D.) over 10 randomly selected fields. After 13-day culture period, merozoites were harvested from all flasks by rinsing the monolayer twice with medium and counting the number of merozoites using a hemocytometer as described.

2.7. SDS-polyacrylamide gel electrophoresis (PAGE) and immunoblotting

Proteins were extracted from an equal number of purified and unpurified merozoites by suspending them in a lysis buffer containing 0.5 M Tris (pH 7.4) with 10% sodium dodecyl sulfate (SDS), 20% glycerol and 5% B-mercaptoethanol and heating the mixture at 95 °C for 5 min. Solubilized protein extracted from approximately 10^7 pure merozoites were loaded onto a PAGE gel in 2, 5, and 10 μl aliquots. For comparison, a 15 μl of solubilized protein extracted from approximately 10^7 crude merozoites was also loaded onto a lane of the same gel. The gel and a biotinylated molecular weight standard (Bio-Rad, Hercules, CA) were separated electrophoretically on a 12–18% SDS-PAGE gradient minigel at 150 V and transferred to Westran PVDF membrane (Bio-Rad). The remainder of the immunoblotting procedure was performed as described previously (Mansfield et al., 2001). Also, the protein extracts were visualized by silver staining as follow; after SDS-PAGE, gels with the separated

proteins were silver stained by fixation for 30 min in 50% methanol–10% acetic acid followed by staining using the Silver Stain Plus kit (Bio-Rad, Hercules, CA), and scanned using a Microtek ScanMaker III (Microtek Lab, Redondo Beach, CA). Protein bands from each lane were compared to size standards and patterns of each lane were compared.

2.8. Immuno-fluorescence assay (IFA)

To prepare IFA slides, serial dilutions of the pure and unpure merozoite stocks were placed in duplicate in wells of a 12-well slides (20 μl/well), air dried and fixed in acetone. Cerebrospinal fluid (CSF) from a horse previously tested positive for *S. neurona* antibodies was placed on each well and incubated for 30 min at 37 °C. The slide was washed several times with PBS and then incubated with fluorescein isothiocyanate (FITC) labeled goat-anti-horse IgG-h+1 secondary antibodies in PBS and 0.1% Evans blue and incubated at 37 °C for an additional 30 min. The slide was washed with PBS, cover-slipped and the optimum dilution of merozoites for IFA slides determined. The stock merozoite solution was diluted with PBS to the optimum and multiple slides prepared through the fixation step and stored frozen at −20 °C until used. Positive sera were applied at 1:2500 and 1:10,000 dilutions in PBS and incubated for 30 min at 37 °C followed by washing as above. Serum from a knockout mouse previously infected with *S. neurona* was used as a positive control. FITC-labeled goat-anti-mouse IgG-h+1 secondary antibody (Kirkegaard and Perry Labs Inc., Gaithersburg, MD, USA) diluted 1:10 in PBS and 0.1% Evans blue dye (as a general protein counterstain) was applied. Stained merozoites were observed by epifluorescent microscopy using the SPOT RT slider ''F'' mount camera (model no. 2.3.1, Diagnostic Instruments Inc., Sterling Heights, MI, USA) and SPOT RT software V3.3.

Effect of purification on the sensitivity or minimum detection limit of *S. neurona* merozoites by IFA assay was evaluated qualitatively by comparing the performance of IFA assay using pure and non-pure parasite materials. Speed and accuracy of *S. neurona* detection as well as sharpness, resolution, and clarity of the microscopic field were the parameters that we used in the assessment of IFA sensitivity.

2.9. Diagnostic sensitivity of conventional PCR for S. neurona

Crude merozoites and merozoites purified using a PD-10 column were diluted and adjusted to equal concentations of 1000 merozoites/200 μl 1× PBS. Ten-fold serial dilutions (1000, 100, and 10 merozoites) from both samples were prepared to a final volume of 200 μl in 1× PBS for DNA extraction using the QIAamp® DNA Blood Mini Kit (Qiagen, Valencia, CA, USA). The Qiagen® Blood and Body Fluid Spin Protocol was followed according to the manufacturer's recommendations to obtain a high DNA concentration from each dilution. DNA concentration was assessed using a NanoDrop DN-1000 spectrophotometer and NanoDrop software 2.5.4. (NanoDrop Technologies, Rockland, DE, USA). The DNA extracts were subjected to polymerase chain reaction (PCR) using primers JNB25 and JD396 (Tanhauser et al., 1999). PCR products were electrophoresed through a 1.8% agarose gel for detection of a 334 bp band, indicative of *S. neurona*.

2.10. Purification of B. darlingi tachyzoites

B. darlingi tachyzoites were purified and analyzed from cultured BT cells using procedures identical to those described for *S. neurona* merozoites. For each purification method used, recovery, purity, and viability of *B. darlingi* tachyzoites were measured using methods identical to those used for *S. neurona* merozoites.

2.11. Quantitative PCR assay design

We developed a new quantitative PCR assay employing specific amplification of a small portion of nuclear 18S rDNA using primers complementary to sequences conserved among coccidia but distinct from other taxa. Additional selection criteria included minimal non-specific annealing, low GC content, balanced primer length, and an amplification product which required fewer than 20 cycles in order to exceed threshold fluorescence (C_t) and dissociated over a narrow and reproducible temperature range. After evaluating several candidate primer combinations, we chose one pair that best met these criteria. Similar

Table 1
qPCR primers

Target	bp	Forward primer	Reverse primer
Coccidian 18S rDNA	230	18S 9F-GTTGGTTTCTAGGACTGA	18S 10R-AGCATGACGTTTTCCTATCTCTA
Vertebrate 18S rDNA	240	V18SF-CTCTTTCGAGGCCCTGTA	V18SR-GGGACACTCAGCTAAGAGCA
Besnoitia darlingi hsp70	212	hsp70F-GGTGAGGCCGCAAARAAYGA	hsp70R-ATGGCGGAGACYTCYTCGG

criteria were used in developing quantitative assays for *B. darlingi* based on the Heat Shock Protein 70 gene (hsp70) and for vertebrate 18S rDNA (Table 1).

Brilliant® SYBR® Green QPCR Master Mix (Stratagene) was used to quantify the accumulation of double-stranded DNA products in a Stratagene Mx300P Real Time PCR System. Melting curves were performed to assure that the fluorescence was derived from dye intercolating into a specific, homogeneous amplification product. Individual 50 μl reactions contained 25 μl of SYBR® Master Mix, 19 μl of ddH20, 1 μl of primer mixture comprising 100 nM of each primer, and 5 μl of template. Subsequent reactions were performed using identical reagent concentrations in a total volume of 25 μl.

To establish that each assay for parasite DNA was quantitative, serial five-fold dilutions of the purified parasite extracts were amplified. Validation of the assay targeting vertebrate rDNA made use of serial dilutions of crude preparations. Although the absolute concentration of parasite DNA in our reference sample was initially unknown, we deemed quantitative those assays whose linear regression of C_t against relative concentration had an R^2 value of >0.90 and an estimated efficiency between 90% and 110%. Assay reproducibility was determined through replication among extractions and among triplicate qPCR assays of individual extracts.

A 10 min pre-incubation at 95 °C was followed by 40 cycles of 30 s at 95 °C, 1 min at 55 °C, and 1 min at 72 °C. The subsequent dissociation step included 1 min incubation at 95 °C, ramping down to 55 °C at a rate of 0.2 °C/s, followed by 81 cycles of incubation where the temperature was increased to 95° by 0.5 °C/cycle every 30 s. Fluorescence was quantified at the end of the annealing, extension, and dissociation steps in each cycle. Each reaction was performed in triplicate to ensure reproducibility. Three wells were used as negative controls; these lacked template but contained all other reagents. The cycle at which fluorescence significantly exceed baseline levels, C_t, was determined using the normalized fluorescence option of the instrument software.

To assess the affect of purification on the effective concentration of parasite DNA targets using qPCR, we first assayed extracts containing 1000 merozoites/200 μl, as estimated by ocular haemocytometry. By normalizing a second set of pre- and post-purification templates to a 1 ng/μl, as estimated by UV absorbance spectroscopy, we then assessed how purification affected the relative proportion of total DNA attributable to parasite and host sources. These estimates, when related to the total DNA concentration estimated from UV absorbance spectroscopy, afforded the means to infer absolute concentrations of host and parasite DNA concentrations before and after column purification. Finally, triplicate assays were performed on mixtures of *B. darlingi* cultures whose purified content comprised 25%, 50% and 75% of the total volume.

2.12. Statistical analysis

For determining recovery, parasite production, and plaque forming efficiency, analysis of variance (ANOVA) procedure was performed using SAS PC version 8 (SAS Institute, Cary, NC, USA). Statistical significance was assigned to P-values <0.05. Charts were made using the graphical software SigmaPlot version 8 (RockWare Inc., Golden, CO, USA).

3. Results

We purified *S. neurona* merozoites and *B. darlingi* tachyzoites by eluting parasite suspensions through PD-10 columns. Such purification was easy to perform and successfully removed visible host cell contaminants from culture media (Fig. 2). By counting both merozoites/tachyzoites and debris particles, we

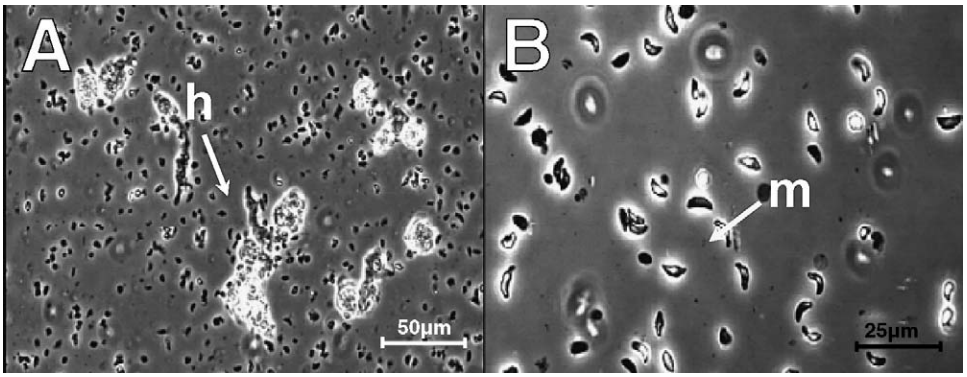

Fig. 2. Phase-contrast photomicrographs of *Sarcocystis neurona* merozoites. (A) Crude merozoite preparations before purification. (B) Merozoites (m) completely free of host cell (h) contaminants and extraneous debris after purification using PD-10 column.

estimated the mean purity of *S. neurona* merozoites and *B. darlingi* tachyzoites at 97.5% and 96.5%, respectively. We also purified the *S. neurona* merozoite and *B. darlingi* tachyzoite suspensions using cellulose filter pads and filter paper. The percentage purity was estimated at 76% and 84% for *S. neurona* merozoite and 83%, 88% for *B. darlingi* tachyzoites, respectively. Even though, the amount of host cell contamination observed in the filter paper-purified parasite fraction was relatively small, purification by filter pads and filter papers reduced the yield to 40% and 30% for *S. neurona* merozoites and 51% and 35%, for *B. darlingi* tachyzoites. In contrast, ~82% of merozoites and 94% of tachyzoites of the original suspensions applied to the columns were recovered (Fig. 3). Occasionally, merozoite yields were lower when the filter pads or filter papers were washed with PBS instead of EMEM, perhaps because the serum in EMEM retarded or prevented merozoite/tachyzoite attachment to the filter pads or filter papers while washing.

We investigated the effect of each purification method on the viability and rate of in vitro development of parasites and by trypan blue dye exclusion assays and by evaluating the rate of parasite production and plaque forming efficiency. Using the trypan blue dye exclusion assay, the estimated viability of *S. neurona* merozoites and *B. darlingi* tachyzoites before filtration was not less than 99%; only very minor reductions in viability were observed in column-purified *S. neurona* (97.6% ± 1.1) and column-purified *B. darlingi* tachyzoites (97.2% ± 0.8).

In all methods, the purified organisms were found to have variable abilities to penetrate BT cells and to reproduce. However, the parasite production of column-purified parasites was significantly better than in parasites purified by filter pad or filter paper (13.2 versus 7 and 5.5; $P = 0.003$ and 0.001, respectively, d.f. = 39 in case of *S. neurona* and 11.4 versus 5 and 6.2; $P = 0.007$ and 0.008, respectively, d.f. = 39 in case of *B. darlingi*). Additionally, the plaque forming ability of column-purified parasites was significantly greater than that for parasites purified by filter pad or filter paper (38 versus 22.7 and 15.6, $P = 0.01$ and 0.009; d.f. = 39 for *S. neurona* and 54 versus 19 and 25; $P = 0.002$ and 0.006, respectively, d.f. = 39 for *B. darlingi*). On the contrary, no significant difference was observed between the crude parasite and column-purified parasites ($P < 0.86$). Cultured equine dermal cells (ATCC, CCL-57) were also used as a source of *S. neurona* merozoites subjected to these three purification methods. The results were similar to those of BT cells in terms of recovery, purity, and viability (data not shown). Likewise, *Toxoplasma gondii* and *Neospora caninum* tachyzoites were also purified using the PD-10 column method and purity percentage obtained in each case was not less than 95% with very minimal loss in tachyzoite recoveries.

The efficacy of *S. neurona* merozoite purification using a PD-10 column was also evaluated by SDS-PAGE and western immunoblotting and silver staining assays. Similar but more intense electrophoretic profiles were obtained from proteins extracted from column-purified merozoites as compared to crude *S.*

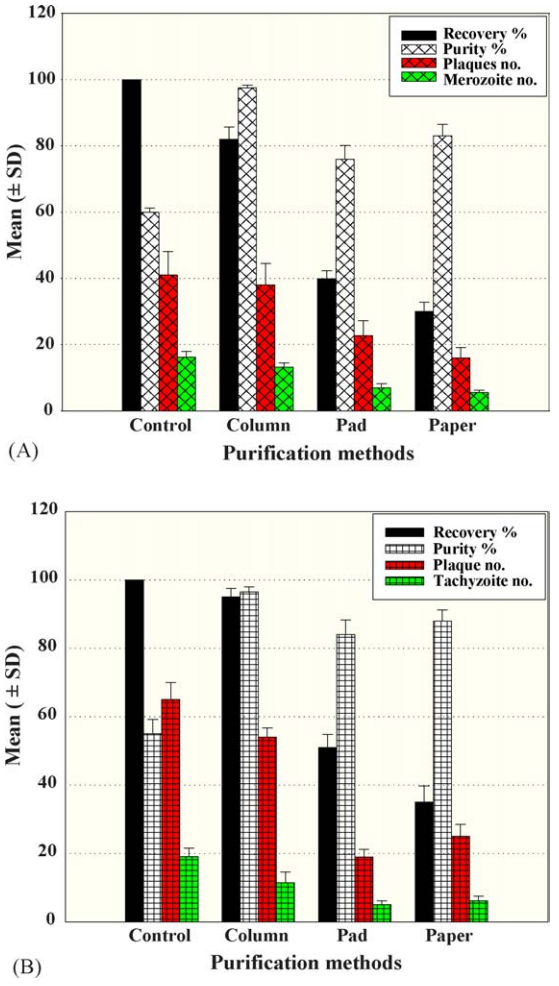

Fig. 3. Purification of *S. neurona* merozoites (A) and *Besnoitia darlingi* tachyzoites (B) cultured in bovine turbinate cells using PD-10 columns, filter pads, and filter papers compared to unpure (crude) merozoites. Number of parasites is expressed as $\times 10^6$. All experiments were conducted in triplicate.

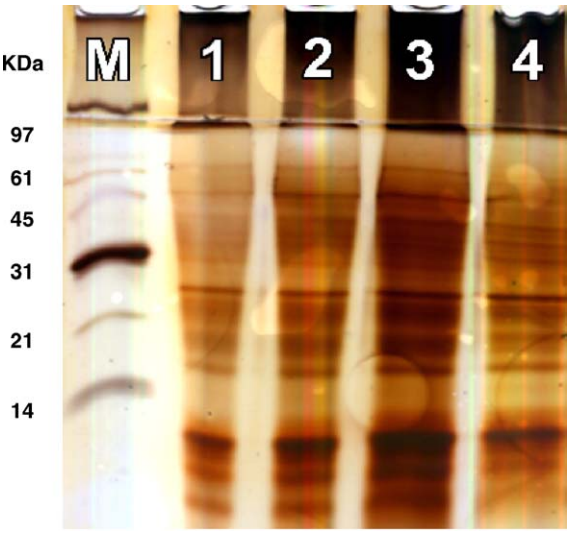

Fig. 4. Silver-stained SDS-PAGE gel of whole-cell protein preparations of *S. neurona* merozoites showing differences in the protein profiles of crude *S. neurona* merozoites cultured in BT cells and PD-10 column-purified merozoites. M, molecular weight markers (Broad Range Standards; Bio-Rad); lanes 1–3 contain the following amounts of *S. neurona* protein extract of column-purified merozoites: (1) 2 μl; (2) 5 μl; (3) 10 μl. Lane 4 contains 15 μl of *S. neurona* protein extract of unpure merozoites. The numbers on the left indicate the molecular masses of proteins in kilodaltons (kDa) used as standard markers.

neurona merozoite preparations (Fig. 4). Western blot analyses revealed ∼30 and 16 kDa antigens in all tested preparations.

The immuno-fluorescent staining (IFA) revealed a marked gain in sensitivity and reduction in background in purified parasite preparations. Using column-purified parasites as compared to non-purified materials markedly expedited the *S. neurona* detection, improved the IFA accuracy of identification, and enhanced the parasite visualization by decreasing the noise caused by background contamination via removal of most host cell debris. Qualitative assessment of IFA sensitivity using pure and non-pure parasite specimens, not surprisingly, suggested that the sensitivity of the method increased roughly in pure specimens in proportion to the extent to which host cell contaminants must be removed to overcome background inhibition. Thus, the use of pure parasites may be necessary to improve the efficiency of IFA sensitivity.

DNA extracts of column-purified *B. darlingi* merozoites and *S. neurona* tachyzoites absorbed

Table 2
Total DNA concentration (ng/μl) in purified and unpurified preparations of DNA estimated to each contains 1000 merozoites/200 μl estimated by UV absorbance spectroscopy

	Before purification	After purification
Sarcocystis neurona	74.7 ± 17.93	4.5 ± 0.50
B. darlingi	15.83 ± 0.12	4.37 ± 0.31

Table 3
Total and parasite DNA content of defined admixtures of crude and column-purified preparations of *B. darlingi* estimated by UV absorbance and qPCR targetting parasite rDNA

Pure:unpure	Observed (ng/μl)	Expected (ng/μl)[a]	Observed, C_t	Expected, C_t[b]
0:4	15.7–15.9	15.8	15.01–15.09	NA
1:3	12.0–12.7	12.94	14.63–14.85	14.86
2:2	9.4–12.3	10.08	14.07–14.47	14.69
3:1	6.0–7.6	7.22	14.00–14.40	14.54
4:0	4.1–4.7	4.36	13.83–14.22	14.40

[a] Assuming purified and unpurified contain 218 and 790 ng DNA, respectively.
[b] Assuming purified contain 1.57 times as much parasite DNA as crude.

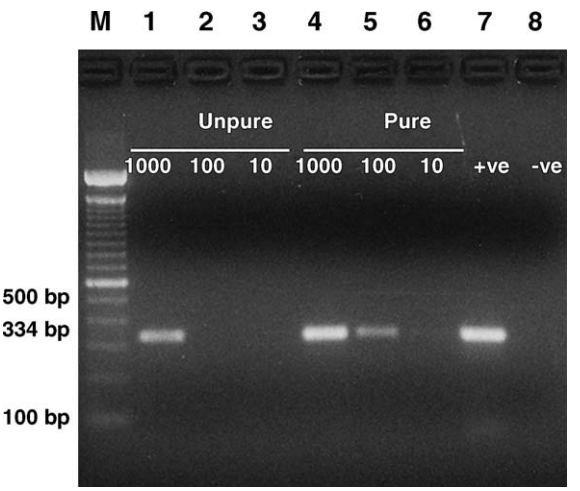

Fig. 5. Analytical sensitivity of PCR analyses using the diagnostic primers JNB25/JD396 on DNA obtained from crude *S. neurona* merozoites cultured in BT cells and PD-10 column-purified *S. neurona* merozoites. Lanes: M, molecular size marker of a 100 bp ladder; lanes 1–3: unpure merozoites (1000, 100, 10, respectively); lanes 4–6: column-purified merozoites (1000, 100, 10, respectively); lane 7: a positive control; lane 8: a negative control. Numbers on the right and on the left of the gel are DNA fragment sizes (in base pairs).

markedly less light at 260 nm than did extracts of crude parasite preparations (Table 2). This absorbance reduction, if caused solely by a reduction in DNA, would indicate a 75% and 90% reduction in the total DNA content of purified *B. darlingi* and *S. neurona* extracts. In mixtures of purified and unpurified *B. darlingi* preparations, the total DNA concentration declined proportionately with the contribution of unpurified merozoites; purified and unpurified extracts from ∼250 merozoites yielded an estimated 218 and 790 ng of DNA, respectively (Table 3). These estimates may overstate the purification-induced reduction in DNA if crude preparations contain disproportionate amounts of contaminants that also absorb light at 260 nm.

We preliminarily tested the effect of column purification on the detection of parasite DNA by performing PCRs on extracts obtained from serial dilutions of purified and unpurified *S. neurona* merozoites and attempting to visualize the resulting product on ethidium bromide-stained agarose gels. PCR primers specific for *S. neurona* amplified only the DNA isolated from the parasite and a diagnostic band of 334 bp was detected from both purified and unpurified parasite templates. Even though the total DNA obtained from 1000 crude *S. neurona* merozoites was estimated at ∼1.2 times that obtained from 1000 pure *S. neurona* merozoites, solely column-purified extracts supported PCR amplification of a diagnostic marker when such extracts were diluted 10- and 100-fold (Fig. 5).

To more precisely quantify how parasite DNA abundance and purity was affected by column purification, we developed three new quantitative real-time PCR assays. Standard curves prepared from five-fold serial template dilutions demonstrated an inverse linear relationship between target DNA concentration and the number of amplification cycles necessary to significantly exceed background fluorescence (C_t) (Fig. 6A). For assays targeting parasite rDNA and hsp70, this relationship remained linear even when purified extracts were diluted to 0.00032 of their original concentration. For the assay targeting vertebrate rDNA, the assay remained linear at dilutions as low as 0.0016 of that present in the original crude extract. Repeated application of these assays resulted in estimated efficiencies of 94.6–110.6%, and R^2 values of 0.957–0.999. Thus, each assay supported template quantification over a broad concentration range.

We first employed qPCR assays to assess how column purification affected the ability to detect host 18S rDNA. Column purification reduced by more than 130-fold the amount of host rDNA detectable from extracts of 1000 *B. darlingi* merozoites. A more modest reduction in host DNA, of approximately

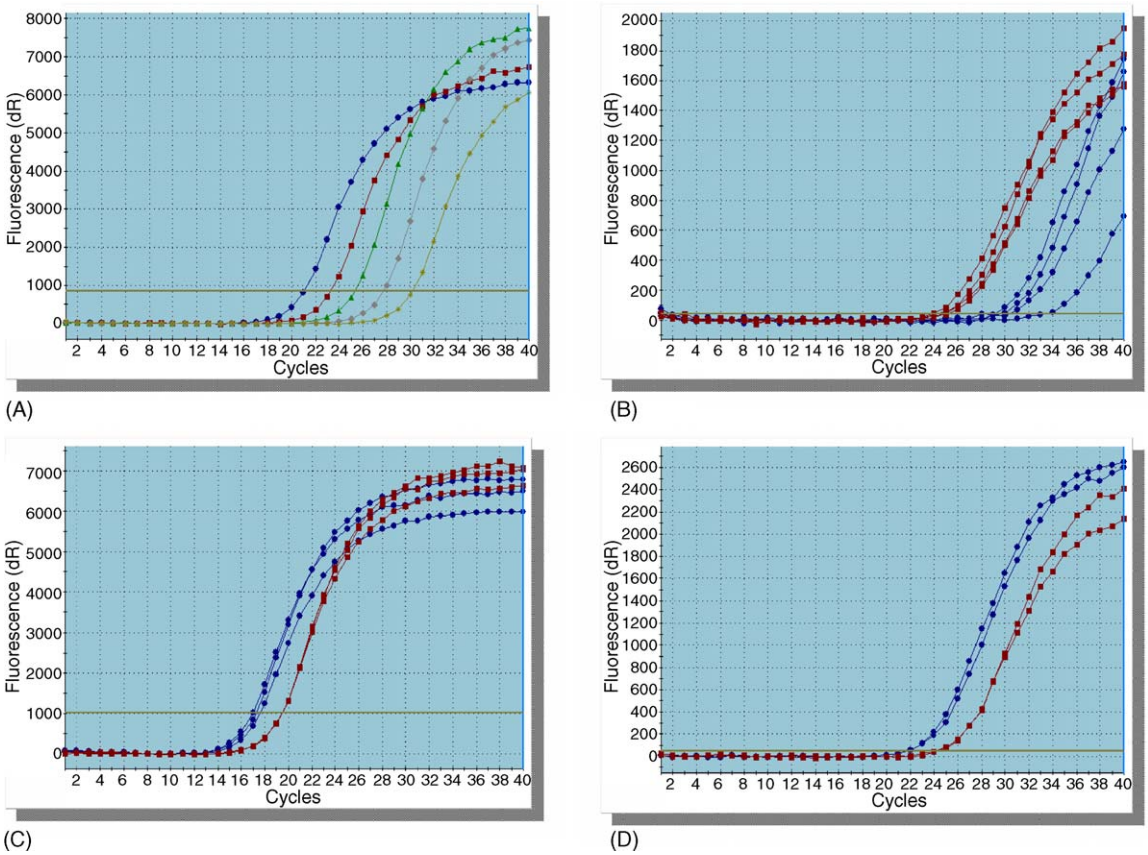

Fig. 6. Amplification plots (fluorescence vs. cycle number) in quantitative PCR assays. (A) Proportional delay in the amplification of parasite rDNA as crude *B. darlingi* are diluted, in serial five-fold steps, over four orders of magnitude. After normalizing crude (red) and column-purified (blue) extracts to a total DNA concentration of 1 ng/μl, amplification of host 18S rDNA is delayed (B) whereas amplification of *B. darlingi* 18S rDNA (C) and *B. darlingi* hsp70 (D) is accelerated.

two-fold, was observed when crude and column-purified extracts of *S. neurona* were compared (Table 4). When total DNA was first normalized to an estimated 1 ng/μl, purification reduced the amount of host rDNA in *B. darlingi* preparations by an average of 55-fold (Fig. 6B) but did not consistently reduce the amount of host rDNA in *S. neurona* preparations.

Column purification consistently permitted earlier detection of parasite rDNA amplification products. A 0.79 and 0.65 reduction in C_t was observed for *B. darlingi* and *S. neurona*, respectively, representing approximately 1.7- and 1.6-fold increases in the effective concentration of each template. An independent assay targeted to the hsp70 gene of *B. darlingi* resulted in a similar detection improvement subsequent to column purification (Table 5).

When the total DNA concentration was first normalized to 1 ng/μl, significantly earlier detection

Table 4
Effect of column purification on the detection of host rDNA amplification in preparations estimated to contain 1000 merozoites/200 μl

	C_t before purification	C_t after purification	ΔC_t	Fold difference
S. neurona	21.74 ± 0.387	22.66 ± 0.301	0.92	1.89
B. darlingi	22.81 ± 0.270	29.88 ± 0.112	7.07	134.36

Table 5
Effect of column purification on the detection of parasite rDNA amplification in preparations estimated to contain 1000 merozoites/200 μl

	C_t before purification	C_t after purification	ΔC_t	Fold difference[a]
S. neurona	16.15 ± 0.185	15.36 ± 0.318	0.79	1.73
B. darlingi	15.04 ± 0.051	14.39 ± 0.187	0.65	1.57

C_t: the critical threshold value is the number of cycles required for fluorescence to significantly exceed background.
[a] Assuming PCR product doubles with each cycle in the exponential phase.

Table 6
Effect of column purification on the detection of parasite rDNA in 1 ng/μl total DNA

	C_t before purification	C_t after purification	ΔC_t	Fold difference[a]
S. neurona	21.42 ± 0.330	17.17 ± 0.164	4.25	19.02
B. darlingi	18.34 ± 0.037	16.16 ± 0.427	2.18	4.53

[a] Assuming PCR product doubles with each cycle in the exponential phase.

of parasite amplification products occurred in purified samples than in crude samples, irrespective of the locus targeted (Fig. 6C and D). The rDNA of purified *B. darlingi* and *S. neurona* reached the critical fluorescence in such normalized templates 2.18 and 4.25 cycles earlier, representing approximately 4.5-fold and 19-fold increases in the representation of parasite DNA (Table 6).

Finally, qPCR assays were performed on defined admixtures of purified and unpurified *B. darlingi* templates in order to determine whether incremental improvements in PCR sensitivity resulted when the proportion of purified preparations was increased. Incremental increases in the relative proportion of purified parasites caused parasite DNA targets to be detected earlier (Table 3).

4. Discussion

Accumulated host cell contaminants can hamper the study of intracellular coccidia. Although purification methods for *S. neurona* merozoites have been previously described (for example those using syringe filters, 3 μm polycarbonate filters, and calcium ionophores) no highly efficient method suitable for routine processing has been reported for the separation of *S. neurona* merozoites or *B. darlingi* tachyzoites from tissue culture cells. We are aware of no previous attempts to quantify the affect of such purification methods on the yield and purity of parasite DNA.

We have found a simple purification method, described initially for *N. caninum* by Hemphill et al. (1996), to be suitable for separating *S. neurona* merozoites and *B. darlingi* tachyzoites from cultured and have favorably evaluated the method according to parasite: (i) yield and purity, (ii) viability, (iii) SDS-PAGE silver staining and immunoblotting, (iv) immuno-fluorescent staining, and (v) quantitative PCR.

Our newly developed quantitative PCR assays help evaluate the yield and purity of such parasite preparations. The improved detection of parasite DNA in column-purified preparations may have been caused by the removal of inhibitory substances, the enrichment of parasite-derived DNA as a proportion of the total, or differences in the accuracy of enumerating merozoites in purified and unpurified preparations. Whatever the cause, parasite targets were more readily amplified after column purification. In the case of *B. darlingi* preparations, contamination by host genomic DNA was markedly reduced as a result of column purification.

The ability to obtain a pure homogenous population of viable *S. neurona* merozoites and *B. darlingi* tachyzoites from large-scale culture facilitates the biological, biochemical, immunological, and genetic studies of these specific stages of those parasites life cycle. This technique appears to have general applicability for purifying coccidia from various cultured cell lines.

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